

Process for preparing recombinant heterocarpine

A subject of the invention is a process for preparing recombinant heterocarpine.

Heterocarpine is a protein with anti-cancer properties described for the first time by the Applicant in Patent Application PCT WO 02/068461. This isolated protein has a molecular mass of approximately 90.9 kDa, comprises fragments of peptide sequences

5 SEQ. ID. NO. 1, SEQ. ID. NO. 2 and SEQ. ID. NO. 3 (see the part of the description reserved for the sequence listing) and is capable of being obtained by extraction from *Pilocarpus heterophyllus* plant cells cultured *in vitro* as described in the abovementioned patent application. However, the complete sequence of this protein remained unknown up to now, to the extent that cloning had not been carried out.

- 10 The present application now describes polynucleotides which can serve as a primer for cloning heterocarpine, the DNA encoding for heterocarpine, the mRNA corresponding to heterocarpine, expression vectors containing said mRNA, host cells transformed or transfected with these vectors as well as a process for preparing recombinant heterocarpine.
- 15 A subject of the present invention is therefore firstly an isolated polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 8. Preferably, said isolated polynucleotide consists of the polynucleotide sequence SEQ. ID. NO. 8.

A subject of the present invention is also an anti-sense polynucleotide comprising the sequence complementary to that of said isolated polynucleotide comprising the 20 polynucleotide sequence SEQ. ID. NO. 8. Preferably, said anti-sense polynucleotide consists of the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 8.

The invention also relates to an isolated polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 8 or one of the fragments of the latter, said polynucleotide 25 being such that it encodes a polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation. Preferably, said isolated polynucleotide is such that it consists of the polynucleotide sequence SEQ. ID. NO. 8 or one of the fragments of the latter.

In particular, the invention therefore relates to the isolated polynucleotide of nucleotide sequence SEQ. ID. NO. 9 or the isolated polynucleotide of nucleotide sequence complementary to the nucleotide sequence SEQ. ID. NO. 9.

Heterocarpine, in other words the protein of sequence SEQ. ID. NO. 10, is encoded by
5 the fragment of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 contained between the bases at positions 115 (initiation codon ATG encoding for a methionine) and 2437 (stop codon UAA), i.e. by the polynucleotide sequence SEQ. ID. NO. 9.

The invention therefore also relates to an expression vector comprising an isolated
10 polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 8 or one of the fragments of the latter or the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 8 or one of the fragments of the latter, the polypeptide encoded by said isolated polynucleotide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the
15 modulation of cell proliferation. Preferably, said expression vector will comprise the polynucleotide sequence SEQ. ID. NO. 9 or the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9.

The invention similarly relates to a host cell transformed or transfected with said expression vector.

20 The invention also relates to an isolated polypeptide comprising a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation. Preferably, said isolated polypeptide will consist of a polypeptide of sequence SEQ. ID.
25 NO. 14 or one of its fragments. In particular, said polypeptide will have the sequence SEQ. ID. NO. 14.

A subject of the invention is also a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds an isolated polypeptide as described previously, and in particular a monoclonal antibody, or an antigen-binding fragment of
30 the latter, which specifically binds the protein of sequence SEQ. ID. NO. 14 but not the protein of sequence SEQ. ID. NO. 10.

The invention also relates to, as a medicament, an isolated polynucleotide comprising:

- the polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments, or

- the polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments, said isolated polynucleotide being such that it encodes an isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation.
- 5 Preferably, the isolated polynucleotide will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments, or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments.
- In particular, said isolated polynucleotide will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9.
- 10 Moreover, said isolated polynucleotide used as a medicament is preferably present in a viral vector, said viral vector being for example selected from the group consisting of an adenovirus, an associated adenovirus, a retrovirus and a pox virus.
- The invention also relates to, as a medicament, an isolated polypeptide comprising a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation. Preferably, said isolated polypeptide will consist of a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments. In particular, said polypeptide will have the sequence SEQ. ID. NO. 14.

15 The invention also relates to, as a medicament, a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds an isolated polypeptide comprising the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation. Preferably, said monoclonal antibody or said fragment of the antigen of the latter specifically binds an isolated polypeptide consisting of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter. Yet more preferentially, said monoclonal antibody or said fragment of the antigen of the latter specifically binds an isolated polypeptide consisting of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by a sequence

complementary to the polynucleotide sequence SEQ. ID. NO. 9 (in other words the protein of sequence SEQ. ID. NO. 10).

Another subject of the invention is a pharmaceutical composition comprising, as active ingredient, an isolated polynucleotide comprising:

- 5 - the polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments, or
 - the polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments,
- said isolated polynucleotide being such that it encodes an isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation,
- 10 with one or more pharmaceutically acceptable excipients.

Preferably, the isolated polynucleotide serving as active ingredient will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments.

- 15 In particular, the isolated polynucleotide serving as active ingredient will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9.

- 20 Said isolated polynucleotide incorporated into a pharmaceutical composition according to the invention is preferably present in a viral vector, said viral vector being for example selected from the group consisting of an adenovirus, an associated adenovirus, a retrovirus and a pox virus.

- 25 The invention also relates to a pharmaceutical composition comprising an isolated polypeptide comprising a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation. Preferably, said isolated polypeptide will consist of a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments. In particular, said polypeptide will have the sequence SEQ. ID. NO. 14.

- 30 A subject of the invention is moreover a pharmaceutical composition comprising a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds an isolated polypeptide comprising at least one fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human

GHRH and being associated with the modulation of cell proliferation, said composition comprising moreover one or more pharmaceutically acceptable excipients. Preferably, said pharmaceutical composition according to the invention is such that it comprises a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds an isolated polypeptide consisting of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter.

In particular, the invention relates to a pharmaceutical composition comprising a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 (in other words the protein of sequence SEQ. ID. NO. 10) with one or more pharmaceutically acceptable excipients.

Another subject of the invention is the use of an isolated polynucleotide comprising:

- the polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments, or
- the polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments,

said isolated polynucleotide being such that it encodes an isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation,

for preparing a medicament intended to treat a proliferative disease.

Preferably, the isolated polynucleotide used will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or one of its fragments or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9 or one of its fragments.

In particular, the isolated polynucleotide used will consist of the polynucleotide of polynucleotide sequence SEQ. ID. NO. 8 or the polynucleotide of polynucleotide sequence SEQ. ID. NO. 9.

Said isolated polynucleotide used is preferably present in a viral vector, said viral vector being for example selected from the group consisting of an adenovirus, an associated adenovirus, a retrovirus and a pox virus.

The present invention also relates to the use of an isolated polypeptide comprising a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments, said isolated polypeptide having at least one immunological and/or biological activity characteristic

of a protein binding human GHRH and being associated with the modulation of cell proliferation, for preparing a medicament intended to treat a proliferative disease. Preferably, said isolated polypeptide will consist of a polypeptide of sequence SEQ. ID. NO. 14 or one of its fragments. In particular, said polypeptide will have the sequence
5 SEQ. ID. NO. 14.

Alternatively, still according to the present invention, a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds an isolated polypeptide comprising the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide
10 sequence SEQ. ID. NO. 9 or one of the fragments of the latter, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, can be used for preparing a medicament intended to treat a proliferative disease. Preferably, said monoclonal antibody or said fragment of the antigen of the latter will
15 specifically bind a polypeptide consisting of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter.

In particular, a monoclonal antibody, or an antigen-binding fragment of the latter, which
20 specifically binds an isolated polypeptide encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 (in other words, a monoclonal antibody, or an antigen-binding fragment of the latter, which specifically binds the protein of sequence SEQ. ID. NO. 10), can be used for preparing a medicament intended to treat a proliferative disease.

25 According to preferred variants of the abovementioned uses, the proliferative disease to be treated by the polypeptide or the polynucleotide described previously is a cancer. According to yet more preferred variants, the cancer is chosen from the group consisting of prostate cancer, breast cancer, lung cancer (and in particular small-cell lung cancer) and colorectal cancer. Breast cancer and small-cell lung cancer are yet more particularly
30 preferred.

The invention moreover offers a method for preparing an isolated polypeptide comprising the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter,
35 said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the

modulation of cell proliferation, said preparation method comprising the following successive stages:

- (a) culture, under suitable conditions in order to obtain the expression of said polypeptide of a host cell transformed or transfected with an expression vector comprising an isolated polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13, the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 or also one of the fragments of the latter, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, and
- 10 (b) isolation of the polypeptide from the host cell cultures.

Preferably, said preparation method will relate to the preparation of an isolated polypeptide consisting of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or one of the fragments of the latter, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, said preparation method comprising the following successive stages:

- 20 (a) culture, under suitable conditions in order to obtain the expression of said polypeptide of a host cell transformed or transfected with an expression vector comprising an isolated polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13, the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 or also one of the fragments of the latter, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, and
- 25 (b) isolation of the polypeptide from the host cell cultures.

In particular, the object of said method will be the preparation of the isolated polypeptide encoded by the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 or by the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 (in other words, the preparation of the protein of sequence SEQ. ID. NO. 10).

According to a yet more preferred variant of said method, the latter will relate to the preparation of the protein of sequence SEQ. ID. NO. 10 and will comprise the following stages:

- (a) culture, under suitable conditions in order to obtain the expression of said polypeptide of a host cell transformed or transfected with an expression vector comprising an isolated polynucleotide comprising the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13 or the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 or SEQ. ID. NO. 13, said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, and
- 5 (b) isolation of the polypeptide from the host cell cultures.

The present invention also offers a process for identifying compounds capable of binding human GHRH and modulating cell proliferation, which comprises the following successive stages:

- 15 (a) bringing each candidate compound into contact with an isolated polypeptide comprising:
 - either a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9,
 - 20 - or a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 13 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 13,
- under conditions and for a time sufficient to allow the candidate agent to bind to the polypeptide, said isolated polypeptide having at least one immunological and/or 25 biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, and
- (b) detection of the binding of each candidate compound to said polypeptide and identification, from the candidate compounds, of the compounds capable of binding human GHRH and modulating cell proliferation.
- 30 In particular, said method for the identification of compounds capable of binding human GHRH and modulating cell proliferation will comprise, in its stage (a), bringing each candidate compound, under conditions and for a time sufficient to allow the candidate agent to bind to the polypeptide, into contact with the isolated polypeptide encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by the sequence complementary to the

polynucleotide sequence SEQ. ID. NO. 9 (in other words, with the protein of sequence SEQ. ID. NO. 10).

An alternative method for the identification of compounds capable of binding human GHRH and modulating cell proliferation comprises the following successive stages:

5 (a) bringing each candidate compound, under conditions and for a time sufficient to allow the candidate agent and the cell to interact, into contact with a cell capable of expressing an isolated polypeptide comprising:

10 - either a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9,

 - or a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 13 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 13,

15 said isolated polypeptide having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and modulating cell proliferation, and

 (b) determination of the effect of each candidate compound on the cell concentration of polypeptide and identification, from the candidate compounds, of the compounds capable of binding human GHRH and modulating cell proliferation.

20 In particular, said alternative method will comprise, in its stage (a), bringing each candidate compound, under conditions and for a time sufficient to allow the candidate agent and the cell to interact, into contact with a cell capable of expressing the isolated polypeptide encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 (in other words, a cell capable of expressing the protein of sequence SEQ. ID. NO. 10).

25 According to preferred methods of implementing the processes for identifying compounds capable of binding human GHRH and modulating cell proliferation described above, the candidate compounds will originate from small molecule libraries resulting from combinatorial chemistry programmes.

30 The pharmacological properties obtained for the polynucleotides and polypeptides according to the invention make the latter suitable for pharmaceutical use. In fact, the isolated polypeptides comprising:

 - either a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9,

- or a fragment of the protein encoded by the polynucleotide sequence SEQ. ID. NO. 13 or by a sequence complementary to the polynucleotide sequence SEQ. ID. NO. 13,

5 which have at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation, as well as the polynucleotides encoding for said polypeptides, can, according to the invention, be administered to cancer patients in order to slow down the progression of their tumors or to make said tumors regress.

10 In the above methods, the protein binding human GHRH and being associated with the modulation of cell proliferation can in particular be the isolated polypeptide encoded by the polynucleotide sequence SEQ. ID. NO. 9 or by the sequence complementary to the polynucleotide sequence SEQ. ID. NO. 9 (in other words, the protein of sequence SEQ. ID. NO. 10).

15 Finally, the invention relates to the polynucleotides of sequence SEQ. ID. NO. 4, SEQ. ID. NO. 5, SEQ. ID. NO. 11 and SEQ. ID. NO. 12, which can in particular be used as primer in the PCR reactions of heterocarpine cloning.

The different elements mentioned above will become evident to a person skilled in the art having once read the more detailed description of the different features of the invention.

20 **Detailed description of the different features of the invention**

As mentioned above, the present invention is generally directed towards products and methods intended to modulate cell growth and to treat cancer. The present invention is based, in part, on the identification of "sequences associated with the modulation of cell proliferation" which are polypeptide and polynucleotide sequences associated with the 25 modulation of cell proliferation. Such cDNA molecules can be prepared from preparations of RNA or mRNA using standard techniques, such as reverse transcription. Similarly, a protein or a polypeptide associated with differentiation comprises the sequence encoded by an mRNA associated with cell differentiation.

30 The pharmaceutical compositions described here can include one or more polypeptides, nucleic acid sequences and/or antibodies. The polypeptides of the present invention include at least one portion of the protein or a variant of the latter binding human GHRH and being associated with the modulation of cell proliferation. The nucleic acid

sequences of the present invention include a DNA or RNA sequence which encodes at least for a portion of such a polypeptide or which is complementary to such an encoding sequence.

- 5 The antibodies are proteins of the immune system or fragments binding to the antigen of the latter, which are capable of binding a portion of the polypeptides described above.

Polynucleotides, polypeptides and proteins according to the invention:

- A particular subject of the present invention is the polynucleotides of sequence SEQ. ID. NO. 8, SEQ. ID. NO. 9 or SEQ. ID. NO. 13 as well as the polypeptide or the protein 10 of sequence SEQ. ID. NO. 14.

- The invention also comprises the polynucleotides having polynucleotide sequences at 15 least 75%, preferably at least 85% and yet more preferentially at least 90% or even 95%, homologous to the sequences of the polynucleotides described above, in particular to the sequences SEQ. ID. NO. 8, SEQ. ID. NO. 9 and SEQ. ID. NO. 13. This also applies *mutatis mutandis* to the other polynucleotides, polypeptides and proteins forming part of the invention, and in particular to the protein of sequence SEQ. ID. NO. 14.

The degree of homology expressed in% is calculated as follows:

$$100 - 100 \times (N'/N)$$

- with N' representing the number of nucleotides or amino acids modified with respect to 20 the sequence SEQ. ID. NO. 8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, SEQ. ID. NO. 13 or SEQ. ID. NO. 14 and N the number of nucleotides of the sequence SEQ. ID. NO. 8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, SEQ. ID. NO. 13 or SEQ. ID. NO. 14.

- According to the invention, the polynucleotide sequences which encode the 25 polypeptides or proteins of the invention, and fragments or fusion proteins of these polypeptides or proteins, can be used in order to generate recombinant DNA molecules which direct the expression of these polypeptides or proteins, or an active portion of the latter, in appropriate host cells. Alternatively, polynucleotide sequences which hybridize with portions of the polynucleotide sequences according to the invention can also be used in nucleic acid hybridization, Southern blot, Northern blot tests, etc.
- 30 Because of the degeneration of the genetic encode, other DNA sequences substantially encoding for the amino acid sequence of the polypeptides or proteins of the invention can be used for the cloning and expression of said polypeptides or proteins. Such DNA

sequences include those capable of hybridizing the polynucleotide sequences of the polynucleotides of the invention under certain stringency conditions which can be adjusted in several ways. For example, during polymerase chain reaction (PCR), the temperature at which the primers hybridize to the matrix or the concentrations of MgCl₂ 5 in the reaction buffer, can be adjusted. During the use of radiolabelled DNA fragments, or oligonucleotides to probe membranes, the stringency can be adjusted by changing the ionic strengths of the washing solutions or by carefully controlling the washing temperature.

Preferentially, such a homologous nucleotide sequence hybridizes specifically with the 10 sequence complementary to the sequence SEQ. ID. NO. 8, SEQ. ID. NO. 9 or SEQ. ID. NO. 13 under stringent conditions (or conditions of "high" stringency). The parameters defining the conditions of stringency depend on the temperature at which 50% of the paired strands separate (T_m).

For the sequences comprising more than 30 bases, and according to Sambrook et al.. 15 (*Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989), T_m is defined by the equation:

$$T_m = 81.5 + 0.41 \times (\% G+C) + 16.6 \times \log[\text{cations}] - 0.63 \times (\% \text{ formamide}) - (600 / \text{number of bases})$$

For the present invention, the stringency conditions of are called "high" when a 20 hybridization temperature 10°C below T_m and hybridization buffers containing a 6 x SSC (0.9 M sodium chloride and 0.09 M sodium citrate) solution are used. Under such conditions, the polynucleotides of aspecific sequences will not hybridize with the polynucleotide of the sequence complementary to the sequence SEQ. ID. NO. 8, SEQ. ID. NO. 9 or SEQ. ID. NO. 13.

25 Altered DNA sequences which can be used in accordance with the present invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence which encodes the same gene product or equivalent function. The gene product can also contain deletions, additions or substitutions of amino acid residues in the sequences of the proteins of the invention, which result in so-called silent changes, 30 thus producing polypeptides and proteins of equivalent function.

Such amino acid substitutions can be carried out on the basis of the polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipatic nature of the residues involved.

For example, negatively-charged amino acids include aspartic acid and glutamic acid, positively-charged amino acids include lysine and arginine, amino acids with polar groups having close hydrophobicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

- 5 The DNA sequences of the present invention can be modified in order to alter the polynucleotide sequences according to the invention for numerous reasons including in a non-limitative manner alterations which modify the process and the expression of the gene product. For example, mutations can be introduced using techniques well known to a person skilled in the art, for example directed mutagenesis, the insertion of new 10 restriction sites, the alteration of the glycosylations, phosphorylation, etc.

In particular, in certain expression systems such as yeast, the host cell can over-glycosylate the gene product. In such a system, it is preferable to alter the polynucleotide sequences in order to eliminate the glycosylation sites. Within the scope 15 of the disclosure of the present invention modified polynucleotide sequences also figure, linked with heterologous sequences in order to encode a fusion protein. The fusion protein (which can be for example the protein of sequence SEQ. ID. NO. 14) can be modified in order to contain a cleavage site located between the sequence of the protein according to the invention (for example the sequence SEQ. ID. NO. 10) and the sequence of the heterologous protein, such that the sequence of the protein according to 20 the invention can be cleaved from the heterologous part.

Polynucleotides encoding for polypeptides associated with the modulation of cell proliferation:

Any polynucleotide which encodes a polypeptide or a portion or a variant of the latter as described here, binding human GHRH and being associated with the modulation of cell 25 proliferation, is covered by the present invention. Such polynucleotides can be single-strand (encoding or anti-sense) or double-strand and can be DNA (genomic, cDNA or synthetic) or RNA molecules.

The polynucleotides encoding for polypeptides binding human GHRH and being associated with the modulation of cell proliferation can be prepared using any technique 30 available to a person skilled in the art. For example, such a polynucleotide can be amplified *via* a polymerase chain reaction (PCR) from cDNA prepared from cells. For this approach, specific primers can be designed and ordered or synthesized; these primers are based on the sequence of said polynucleotide. An amplified portion can then be used in order to isolate the complete gene of any cell or any tissue from a genomic

- DNA library or from a cDNA library, by means of techniques well known to a person skilled in the art and briefly recalled below. Alternatively, a complete gene can be constructed from several PCR fragments. The cDNA molecules encoding for a protein binding human GHRH and being associated with the modulation of cell proliferation, or
- 5 a portion of the latter, can also be prepared by screening a cDNA library obtained for example from mRNA of cells or tissues. Such libraries can be commercially available or can be prepared using the standard techniques (cf. Sambrook et al., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989).
- 10 Alternatively, other screening techniques well known to a person skilled in the art can be used.

A cDNA molecule encoding for a polypeptide binding human GHRH and being associated with the modulation of cell proliferation can be sequenced by using the standard techniques using enzymes such as the Klenow fragment of DNA polymerase I,

15 Sequenase X (US Biochemical Corp., Cleveland, OH, United States), Taq polymerase (Perkin Elmer, Foster City, CA, United States), thermostable polymerase T7 (Amersham, Chicago, IL, United States) or a combination of recombinant polymerases and exonucleases with a rereading activity such as the Elongase amplification system (Gibco BRL, Gaithersburg, MD, United States). An automatic sequencing system can

20 be used, using instruments available from commercial suppliers such as Perkin Elmer and Pharmacia.

The partial sequence of a cDNA can be used in order to identify a polynucleotide sequence which encodes the complete protein associated with the modulation of cell proliferation using standard techniques well known to a person skilled in the art. Among

25 these techniques, a cDNA library is screened using one or more polynucleotide probes using RecA recombination properties (ClonCapture cDNA Selection Kit, Clontech Laboratories, United States).

For the hybridization techniques, a partial sequence can be radiolabelled (for example by nick translation or labelling of the ends using ^{32}P or ^{33}P) using standard techniques.

30 A bacteria or bacteriophage library is then screened by hybridization on filters containing denatured bacterial colonies (or blots containing phage plates) with the labelled probe (cf. Sambrook et al., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). The positive colonies or plates are then selected and amplified and the DNA is isolated for future analyses.

The complete sequence can then be determined using standard techniques. The overlapping sequences are then assembled into a continuous single sequence. A complete cDNA molecule can be generated by ligation of the fragments of interest using standard techniques.

- 5 Alternatively, numerous techniques based on amplification exist for obtaining a complete encoding sequence from a partial cDNA sequence. Among these, the amplification is generally carried out via PCR. All of the kits commercially available can be used for the amplification stages. The primers can be designed using, for example, software well known in the art. The nucleotide primers are preferably
10 molecules with 20 to 30 nucleotides having a guanine and cytosine content of at least 50% and which hybridize with the target sequence at temperatures comprised between 50 and 72°C. The amplified region can be sequenced as described above and the overlapping sequences assembled into a continuous sequence.

Among the alternative approaches, sequences adjacent to the partial sequence can be
15 found by amplification with a primer of the binding sequence and a primer specific to a known region. The amplified sequences are then subjected to a second amplification cycle.

Additional techniques include capture PCR (Lagerstrom et al., *PCR Methods Applic.* (1991), 1, 111-19) and progressive PCR (Parker et al., *Nucl. Acids. Res.* (1991), 19,
20 3055-60). Other methods using amplification can also be used in order to obtain a complete cDNA sequence.

It is possible to obtain a complete cDNA sequence by analyzing the sequences deposited in the public “Expressed Sequence Tags” (ESTs) bases available from GenBank.
25 Research covering the ESTs can be carried out using computer programmes well known to a person skilled in the art (for example NCBI BLAST) and such ESTs can be used to generate a continuous complete sequence.

Variants of the polynucleotide sequences described above (in particular sequences SEQ. ID. NO. 8, SEQ. ID. NO. 9 and SEQ. ID. NO. 13) are also included within the scope of the present invention. The polynucleotide variants can contain one or more substitutions, deletions or insertions (cf. also above in the part entitled
30 “Polynucleotides, polypeptides and proteins according to the invention”).

A portion of the sequence complementary to the encoding sequence (i.e. an anti-sense polynucleotide) can also be used as a probe or modulator of gene expression. The cDNA construct which can be transcribed to anti-sense RNA can be introduced into

cells or tissues in order to facilitate the production of anti-sense RNA. An anti-sense polynucleotide can be used, as described here, in order to inhibit the expression of a gene associated with the modulation of cell proliferation. The anti-sense technology can be used in order to control gene expression by forming a triple-helix, which 5 compromises the ability of the double helix to open sufficiently for the fixing of polymerases, transcription factors or regulation molecules (cf. Gee et al.. in Huber and Carr, *Molecular and Immunologic Approaches* (1994), Futura Publishing Co., Mt. Kisco, NY). Alternatively, an anti-sense molecule can be used to hybridize with a gene 10 control region (for example a promoter or initiation site of the transcription) and to block the transcription of the gene, or to block the translation by inhibiting the fixing of ribosomes to the transcript.

The polynucleotides can then be modified in order to increase their stability *in vivo*. Possible modifications include (but are not limited to): the addition of sequences to the 15 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase bonds in the backbone; and/or the introduction of bases such as inosine, queosine and wybutoxine as well as acetyladenine, methylthioadenine and modified forms of adenine, cytidine, guanine, thymine and uridine.

Other variations of the polynucleotides of the present invention have moreover already 20 been described previously in the part entitled "Polynucleotides, polypeptides and proteins according to the invention".

The nucleotide sequences as described in the present invention can be joined to other nucleotide sequences using established recombinant DNA techniques. For example, a 25 polynucleotide can be cloned in a large panel of expression vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. The vectors of particular interest include the expression vectors, replication vectors and sequencing vectors. In general, a vector contains a functional replication origin in at least one organism, suitable endonuclease restriction sites and one or more selection markers. The presence of other elements will depend on the specific use desired by a person skilled in the art who will 30 select the characteristics of the expression vector as a function of his requirements and the techniques available.

The polynucleotides can be formulated in order to enter the cell and express the corresponding polypeptide. Such formulations are particularly useful in therapy as described hereafter.

Persons skilled in the art will appreciate that several means exist for expressing a 35 polynucleotide in a target cell, and that any appropriate technique can be used. For

example, a polynucleotide can be incorporated into a viral vector such as an adenovirus or a retrovirus (but also into others). Techniques for incorporating DNA into such vectors are well known to a person skilled in the art. A retroviral vector can transfer or incorporate a gene for a selection marker and/or a screening entity such as a gene encoding for the ligand of a specific receptor of a target cell, in order to render the vector target-specific.

Other formulations for the polynucleotides include colloidal dispersion systems such as macromolecular complexes, nano-capsules, microspheres, spheres, and systems based on the use of lipids including oil/water emulsions, micelles, mixed micelles and liposomes. The preferred colloidal system for use for delivering the product *in vitro* and *in vivo* is the liposome (i.e. an artificial membrane vesicle).

Polypeptides binding human GHRH and modulating cell proliferation:

Within the scope of the disclosure, the polypeptides of the present invention include at least one portion of the protein associated with the modulation of cell proliferation or a variant of the latter, said portion being immunologically and/or biologically active. Such polypeptides can have any length, including the complete protein, an oligopeptide (i.e. consisting of a relatively limited number of amino acids, such as 8-10 residues; joined by peptide bonds) or a peptide of intermediate size. A polypeptide can also comprise additional sequences.

Similarly, a polypeptide is “biologically active” if it has one or more structural, regulatory and/or biochemical functions starting with the native protein associated with the binding of human GHRH and being associated with the modulation of cell proliferation.

The presence of a biological activity can be determined according to processes well known to a person skilled in the art. However, by definition within the framework of the present invention, a polypeptide is considered as “having at least one immunological and/or biological activity characteristic of a protein binding human GHRH and being associated with the modulation of cell proliferation” once its inhibitory concentration IC₅₀ measured under the conditions described in Example 6 of the present Application is less than or equal to 10 nM (and preferably less than or equal to 1 nM).

For example, comparative studies of sequences can indicate a particular biological activity of the protein. Tests for evaluating said activity can then be implemented on the basis of tests already known in the art. Certain portions and other variants of such proteins should also show this property according to an *in vitro* or *in vivo* test.

As already mentioned, the polypeptides according to the present invention can comprise one or more portions of a variant of the endogenous protein where the portion is immunologically and/or biologically active (i.e. the portion has one or more antigenic, immunogenic and/or biological characteristics of the complete protein). Preferably, such 5 a portion is at least as active as the total protein during tests allowing the detection of such properties. A "variant" polypeptide is a polypeptide which differs from the native protein by substitutions, insertions, deletions and/or modifications of amino acids. Certain variants contain conservative substitutions. "A conservative substitution" is a 10 substitution in which one amino acid is substituted by another amino acid having the same properties, such as those determined by a person skilled in the art who expects no change in the secondary structure, as well as in the hydropathic nature of the polypeptide. The amino acid substitutions can generally be carried out on the basis of 15 similarity of polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipathic nature of the residues. For example, negatively-charged amino acids include aspartic acid and glutamic acid; positively-charged amino acids include lysine and arginine; and polar non-charged amino acids having similar hydrophobicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; serine, threonine, phenylalanine and tyrosine. Other amino acid groups which can 20 represent conservative changes are in particular the following: (1) Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr; (2) Cys, Ser, Tyr, Thr (3) Val, Ile, Leu, Met, Ala, Phe; (4) Lys, Arg, His; and (5) Phe, Tyr, Trp, His. A variant can also, or alternatively, contain non-conservative changes.

Variants forming part of this invention also include polypeptides in which the primary 25 structure of the native protein is modified by formation of covalent conjugates or not modified with other polypeptides or chemical structures such as lipid groups or glycosyl, or phosphate acetyl groups.

The present invention also includes polypeptides with or without glycosylation patterns. The polypeptides expressed in expression systems of yeast or mammal cells can, in terms of molecular weight and glycosylation pattern, be similar to or slightly different 30 from the native molecule according to the expression system used.

The expression of DNA in bacteria such as *E. Coli* leads to non-glycosylated molecules. The N-glycosylation sites of the eucaryotic proteins are characterized by the triplet of amino acids Asn-A1-Z where A1 is any amino acid except Pro, and Z is a serine or a threonine.

Other variations of the polypeptides and proteins of the present invention have moreover already been described previously in the part entitled "Polypeptides and polynucleotides according to the invention".

In order to prepare a polypeptide variant, standard mutagenesis techniques, such as
5 directed mutagenesis using a directed oligonucleotide, can be used.

In general, any expression vector known to a person skilled in the art can be used in
order to express recombinant polypeptides of this invention. The expression can be
obtained in any appropriate host cell which has been transformed or transfected with an
expression vector containing a DNA sequence which encodes the recombinant
10 polypeptide. Suitable host cells include prokaryotic, higher eukaryotic or yeast cells.
Preferably, the host cells used are *E. Coli*, yeast cells or mammal cells such as COS,
CHO, HEK-293, MCF7 (human tumor cells isolated from a mammary carcinoma) or
DU 145 (human tumor cells isolated from a prostate cancer).

Certain portions and other variants can also be generated by synthetic means using
15 techniques well known to a person skilled in the art. For example, portions and other
variants having less than 500 amino acids, preferably less than 100 amino acids and
more preferentially less than 50 amino acids can be synthesized by chemical route. The
polypeptides can be synthesized using solid phase synthesis techniques available
commercially, such as the Merrifield resin synthesis method where the amino acids are
20 sequentially added to a chain of amino acids during the synthesis process (cf.
Merrifield, *J. Am. Chem. Soc.* (1963), **85**, 2149-2146). Numerous other solid phase
synthesis techniques are also available (for example the process of Roberge et al.,
Science (1995), **269**, 202-204). Equipment for the automatic synthesis of polypeptides is
commercially available from suppliers such as Applied Biosystems, Inc. (Foster City,
25 CA, United States); the synthesis of the polypeptides can then be carried out by
following the manufacturer's recommendations.

Isolated polynucleotides or polypeptides:

In general, the polypeptides and polynucleotides described in the present invention are
isolated. An "isolated" polypeptide or polynucleotide is a polynucleotide or a peptide
30 removed from its original environment. For example, a natural protein is isolated if it is
separated from the biological material with which it coexists in the natural system. A
polynucleotide is considered as isolated if, for example, it is cloned in a vector which
does not form part of the natural environment.

Antibodies and fragments of the latter:

The present invention provides binding agents, such as the antibodies which specifically bind the protein associated with the binding of human GHRH and being associated with the modulation of cell proliferation. Such an agent is referred to as "specifically binding" to the cell proliferation modulation protein if it reacts at a level which is detectable (for example by an ELISA test) with a protein associated with the modulation of cell proliferation or a portion or a variant of the latter and does not react in detectable manner with other proteins. "The binding" refers to a non-covalent association between 2 separate molecules such that a complex is formed. The binding ability can be evaluated, for example, by determination of the binding constant for the formation of the complex. The binding constant is the value obtained when the value of the concentration of the complex is divided by the product of the values of the concentration of the components. In general, 2 products are called "bound" when the binding constant reaches 103 l/mol. The binding constant can be determined using methods well known to a person skilled in the art.

Any agent capable of fulfilling the above criteria can be considered as a binding agent.

In the present invention, a binding agent is preferably an antibody or a fragment of the latter. The antibodies can be prepared by any technique available to a person skilled in the art (cf. Harlow and Lane, *Antibodies. A Laboratory Manual*, Cold Spring Harbor 20 Laboratory, 1988). In general, the antibodies can be produced by cell culture techniques including the generation of monoclonal antibodies or via transfections of genes of antibodies in host cells of bacteria or mammals in order to produce recombinant antibodies.

Among other techniques, it is preferable to use those described hereafter. An immunogen containing the polypeptide is injected into a group of mammals (for example mice, rats, rabbits, sheep or goats). In this stage, the polypeptides of the present invention can serve as immunogens without modification. Alternatively, and particularly for small peptides, a better immune response can be induced if the polypeptide is joined to a transport protein such as bovine serum albumin or keyhole-limpet hemocyanin. The immunogen is injected into the host animal, preferably according to a predetermined schema, and the animals are bled periodically. Polyclonal antibodies specific to the polypeptide can thus be purified from such antisera, for example by affinity chromatography using the peptide coupled to an appropriate solid support.

In order to prepare an antibody specifically binding a protein of sequence A but not a protein of sequence B, the protein of sequence A is injected into the host animal, preferably according to a predetermined schema, and the animal are bled periodically.

5 Polyclonal antibodies specific to the polypeptide of sequence A can thus be purified from such antisera, for example by affinity chromatography using the protein of sequence B coupled to an appropriate solid support. The corresponding eluate contains the antibody specifically binding a protein of sequence A but not a protein of sequence B.

Fusion proteins:

10 Any fusion gene can be produced by a person skilled in the art in order to analyze the sub-cellular location of a protein according to the invention, in particular the sub-cellular location of the protein of sequence SEQ. ID. NO. 10. Numerous plasmidic constructions are available commercially such as Glutathione S Transferase (GST) protein or fluorescent proteins such as Green Fluorescent Protein (GFP) or also and 15 non-exhaustively a polyhistidine tag.

Human eucaryotic host cells (for example HEK-293) are sub-cultured for 24 hours before the transfection protocol allowing normal metabolism of the cells and better transfection effectiveness. Increasing concentrations (1, 5 and 10 µg) of vector alone containing the developer protein (GFP, GST or Tag Histidine) or of vector containing 20 the polynucleotide of sequence SEQ. ID. NO. 8 or the polynucleotide of sequence SEQ. ID. NO. 9 fused with the developer protein were produced using the reagent Effectene® according to the manufacturer's (Qiagen) recommendations.

The cells are then analyzed by confocal microscopy, for example, in order to detect the 25 location of the protein. If the protein is suspected of being secreted for example, the supernatants are recovered, lyophilized, deposited on acrylamide gel and analyzed by the Western blot technique using antibodies directed against the developer protein.

Pharmaceutical compositions:

According to certain features of the invention, products such as polypeptides, antibodies and/or nucleic acids can be incorporated into pharmaceutical compositions or vaccines.

30 The pharmaceutical compositions comprise one or more of these products and one or more pharmaceutically acceptable excipients (carriers). Certain pharmaceutical compositions optionally usable as vaccines can comprise one or more polypeptides and an immune response activator, such as an adjuvant or liposome (into which the product

is incorporated). The pharmaceutical compositions and the vaccines can moreover contain an administration system, such as biodegradable microspheres (and for example microspheres composed of lactic acid and glycolic acid copolymers or PLGA). The pharmaceutical compositions and the vaccines within the scope of the disclosure of the 5 present invention can also contain other products being able to be biologically active or inactive.

A pharmaceutical composition or a vaccine can contain DNA encoding for one or more polypeptides as described above, such that the polypeptide is generated *in situ*. As mentioned previously, the DNA can be present in any administration form known to a 10 person skilled in the art, including bacterial or viral expression systems of nucleic acids. The appropriate expression systems of nucleic acids contain the DNA sequences necessary for expression in the patient.

The administration systems based on a bacterium involve the administration of a *bacterium* (such as Bacillus-Calmette-Guerrin) which expresses an immunogenic 15 portion of the polypeptide at its surface. Preferably, the DNA can be introduced using a viral expression system (for example a pox virus, retrovirus or adenovirus) involving the use of (defective) non-pathogenic agents.

Although any appropriate carrier known to a person skilled in the art can be used in pharmaceutical compositions of this invention, the type of carrier will vary according to 20 the chosen administration method. The compositions of the present invention can be formulated for each appropriate administration method, including, for example, the topical, nasal, intravenous, intra -cranial, intra-peritoneal, sub-cutaneous and intramuscular routes.

For a parenteral administration, such as a sub-cutaneous injection, the carrier preferably 25 contains water, salt, alcohol, fat, paraffin or a buffer. For oral administration, any carrier mentioned above or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, talc, cellulose, glucose, sucrose and magnesium carbonate can be used. Biodegradable microspheres can also be used as carriers for the pharmaceutical compositions of this invention. For certain topical applications, formulations such as 30 creams or lotions are preferred.

Such compositions can also comprise buffers (for example neutral or phosphate buffered saline solutions), carbohydrates (for example glucose, mannose, sucrose or dextran), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (for example aluminium 35 hydroxide) and/or protective agents. Alternatively, the compositions of the present

invention can be presented in the form of a lyophilisate. Products can also be encapsulated in liposomes using standard technologies.

According to the invention, each of the varieties of adjuvant can be used in vaccines in order to induce the immune response. Most of the adjuvants contain a substance 5 protecting the antigen from a rapid catabolism, such as aluminium hydroxide or mineral oil and an immune response stimulator such as lipid A, proteins derived from *Bordetella Pertussis* or *Mycobacteum tuberculosis*. Appropriate adjuvants are commercially available such as, for example: Freund's adjuvant and complete adjuvant (Difco Laboratories, Detroit, MIY, United States; Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ, United States)), biodegradable microspheres; monophosphoryl lipid 10 A; and cytokines such as GM-CSF or interleukin-2, -7 or -12.

The compositions described above can also be administered in the form of retard formulations (i.e. a formulation such as a capsule or a sponge which triggers the slow release of the product after administration). Such formulations can generally be 15 prepared using technologies well known to a person skilled in the art and administered, for example, by oral, rectal route or sub-cutaneous implantation or implantation at the desired target site. The retard formulations can contain a polypeptide, a polynucleotide or an antibody dispersed in a carrier matrix and/or contained in a reservoir protected by a diffusion membrane. The carriers for the use of such formulations are biocompatible 20 and must also be biodegradable; preferably the formulation provides a relatively constant level of release of the active component. The quantity of active product contained in the retard formulation depends on the implantation site.

Anticancer therapy:

According to other features of the present invention, the products described can be used 25 in anticancer therapy. In particular, the polynucleotides and polypeptides associated with the binding of human GHRH and being associated with the modulation of cell proliferation can be used in order to inhibit growth and induce the modulation of cell proliferation in tumours specific to the breast, the prostate or lung cancer.

Such polypeptides or polynucleotides can also be used for the therapy of numerous 30 carcinomas including melanomas, the multiple forms of glioblastomas, carcinomas of the lung as well as colorectal cancers. Agents which activate the expression of such polypeptides or polynucleotides can also be used within the framework of these therapies.

According to these aspects of the invention, the products (which can be polypeptides or nucleic acids) are preferably incorporated into pharmaceutical compositions as described above.

- Appropriate patients for the therapy are all warm-blooded animals, and preferably 5 human beings. A patient eligible for a therapy according to the invention may or may not be diagnosed as affected by a cancer. In other words, the pharmaceutical compositions described above can thus be used in order to inhibit the development of a cancer at different stages of the disease (in order to prevent the appearance of a cancer or in order to treat a patient affected by a cancer).
- 10 The pharmaceutical compositions of the present invention are administered in a manner appropriate to each specific cancer to be treated.

The route, duration and frequency of administration are determined as a function of the state of the patient, type and severity of the disease, and administration method. The routes and frequencies of administration can vary from one individual to another. In 15 general, the pharmaceutical compositions and the vaccines can be administered by injection (for example by intra-cutaneous, intramuscular, intravenous or sub-cutaneous route), by intra-nasal route (for example by inhalation) or by oral route. Preferably, between 1 and 10 doses can be administered over a 52-week period. Alternative protocols may be appropriate for each patient individually.

- 20 In general, an appropriate dosage and a treatment regime contains the active ingredient in a quantity sufficient to provide a therapeutic and/or prophylactic benefit. Such a response can be followed by the establishment of an improved clinical outlook (for example more frequent remissions, survival in the complete, partial or longer absence of the disease) in patients treated compared with patients not treated or treated with 25 smaller doses.

According to other features of the present invention, a polypeptide can be administered in doses varying from 100 µg to 5 mg. The DNA molecules encoding for such polypeptides can generally be administered in a quantity sufficient to generate comparable polypeptide levels. Appropriate dosages can generally be determined using 30 experimental models and/or clinical tests. In general, the use of the minimum dose sufficient to provide an effective therapy is preferred. The patients can generally be monitored with regard to the effectiveness of the therapy using tests appropriate to the conditions of treatment or prevention which will appear familiar to a person skilled in the art.

Unless otherwise defined, all the technical and scientific terms used here have the same meaning as that commonly understood by an ordinary specialist in the field to which this invention belongs. Similarly, all the publications, patent applications, all the patents and all other references mentioned here are incorporated by way of reference.

- 5 The following examples are presented in order to illustrate the above procedures and should in no case be considered as a limit to the scope of the invention.

EXAMPLES

Example 1: cloning of the cDNA encoding for heterocarpine

1.1) Extraction of the RNAs from *Pilocarpus Heterophyllus* cells:

- 10 The cells in culture are preserved at -80° C before the stages of total RNA extraction. The total RNA extraction is based on a technique described in the scientific literature (Chomczynski and Sacchi, *Anal. Biochem.* (1987), **162**, 156) using Trizol reagent (Gibco/BRL). The quality of the RNAs thus extracted is analyzed on 1% agarose gel in the presence of ethidium bromide.

- 15 1.2) *Synthesis of the cDNAs by reverse transcription:*

The RNAs are retrotranscribed according to two different operating methods in order to dissociate and promote the reverse transcriptions of the 5' and 3' parts of the RNAs using the SMART™ RACE cDNA Amplification Kit (Clontech).

1.3) Design and synthesis of primers for polymerase chain reaction (PCR):

- 20 The amplification of the 2 sequences specific to the cDNA of heterocarpine was carried out by polymerase chain reaction (PCR) on reverse transcription products using the Rev1 primer for the products of 5' specific cDNA and the Fwd1 primer for the products of 3' specific cDNA of respective sequences SEQ. ID. NO. 4 and SEQ. ID. NO. 5.

The sequences SEQ. ID. NO. 4 and SEQ. ID. NO. 5 are the following:

- 25 - SEQ. ID. NO. 4:

5'-TCC AAG CAG CAA AAA CTA GTG ACC CAG GGG CCA TTA TAT CT-3'

- SEQ. ID. NO. 5:

5'-CGG TAT GGA CGC GGC TAT TGC TGA TGG TGT TGA TGT AA-3'

1.4) Polymerase chain reaction (PCR) and results:

The reaction conditions include 0.2 µM of Fwd1 for the products of 3' specific cDNA and 0.2 µM of Rev1 for the products of 5' specific cDNA, 200 µM dNTPs, 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM Mg(OAc)₂, 3.75 µg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, and 0.5 U Taq DNA polymerase in a final volume of 50 µl. The PCR reactions are carried out in a Perkin-Elmer 9700 thermocycler using the following thermal cycle parameters: 5 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 72°C, 5 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 70°C for 10 seconds, and an extension of polymerization at 72°C for 3 minutes and finally 25 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 68°C for 10 seconds, and a polymerase extension at 72°C for 3 minutes.

The products obtained by PCR are separated on 1% agarose gel and visualized using ethidium bromide staining.

The nucleic acid sequences of the products of 5' specific and 3' specific cDNA PCR are determined using an automatic sequencer. These are respectively the sequences SEQ. ID. NO. 6 and SEQ. ID. NO. 7 reproduced hereafter:

- SEQ. ID. NO. 6:

20 1 ctaatacgcac tcactatagg gcaaggcgtg gtatcaacgc agagtacgcg gggatgcccc
61 aagctaattc ttatctttt tctttctttt tgttgttgg ttgtcaaagc agcaatgagg
121 tcttaggaatg gtgttcttca tttattcctt ttcgttcttg catggcttct gttggcggct
181 ctccatgcta actcaagttc ggatgagaga tcaacatata tagttcatat ggacaagacc
241 catatgccca aaaccttctc tagccccac cattggtaact cttcggtcgt tcgatccctc
25 301 aagtctacaa agccaaccaa attaaatcgc cgtcgatcct caccacttct tgtatactct
361 tacgacaatg ctgctcatgg tttcagtgca gtttatctc aacaggaact taaaactcta
421 aaaaagtctc caggttcgt ctcatgttat gccgataaga cagcgacact tgacaccacc
481 catacacctg aatttctctc cctgaataact gccaacgggt tgtggcctgc ttcaaagtat
541 ggtgaagata taattgttgg tgttattgac agcgggtctt ggccggagag taaaagttat
30 601 aatgatgatg gtatggcgc tattccaagc agatggaagg gagaatgtga agctggacaa
661 gagttcaatt cctccatqtq caactcaaaq cttattqqaq ctaqatattt cqataaqqqt

721 atcattgcgg caaatcctgg gattaacatt agcatgaaat ctgccagaga tactatgggg
781 catgggactc acacatcctc cacagttgct gggaaattatg tggatggcgt ttcattctt
841 ggctatgcta aaggtacagc aaaaggagtg gcaccacggg cgagagtggc tatgtacaag
901 gtcatttttg acgaaggcgct ctagcatct gatgttcttgc ccgttatgga cgccgctatt
5 961 gctgatggtg ttgatgtaat ttcaatatca atgggatttg atgagacccc gttgtatgaa
1021 gatcctatacg caattgcctc attcgctgct acagagaagg gcgtagtggt ctcatcttca
1081 gcagggaaatg cagggccagc gctagggagc ttgcacaatg gaatcccattg gacgtaact
1141 gttgcagctg gaaccattga ccgttcattt gcaggcacta taactcttgg gagtggggaa
1201 accatcatttgc gatggacaat gttcccagcc agtgcttatg tagcagactt gccactgctt
10 1261 tataacaaga cttactctgc atgcaactca actcgattat tatctcaact ccgaactgac
1321 gccatcatcg tatgcgaaga agctgaagat tcggtatctg agcaaatac tgggtcagt
1381 gcatcgaaca ttcggggagc catatttttttgc ttagattatg atgctgaatt atttgaactt
1441 ggtgggtgtga ctattcctgg tgctgtgatt agcaccaagg atgcaccggc tgtgatcagc
1501 tacgccagca atgatgtgaa acctaaggca agcatcaagt tccaacaaac tgggttgggg
15 1561 acaaaggcctg caccagccgt ggctttctat acttcttagag gtccgtcacc gagctatcca
1621 ggcatcttaa agccagatataatggcccccttggtcactag ttttgcgtgc ttgga

- SEQ. ID. NO. 7:

1 cggtatggac gcggctatttgc ctgatgggtgt tgatgttaatt tcaatatcaa tgggatttga
61 tgagaccccg ttgtatgaag atcctatacg aattgcctca ttgcgtgcta cagagaagg
20 121 cgtagtggtc tcatcttcag caggaaatgc agggccagcg cttagggagct tgcacaatgg
181 aatcccatgg acgttaactg ttgcagctgg aaccattgac cggttcatttgc caggcactat
241 aactcttggg agtggggaaa ccatcattgg atggacaatg ttcccagcca gtgcttatgt
301 agcagacttg ccactgctt ataacaagac ttactctgca tgcaactcaa ctgcatttatt
361 atctcaactc cgaactgacg ccatcatcg atgcgaagaa gctgaagatt cggtatctga
25 421 gcaaataatct gttgtcagtgc catcgaacat tcggggagcc atatttgttt cagattatga
481 tgctgaatta tttgaacttg gtgggtgtgac tattcctggt gtcgtgatta gcaccaagga
541 tgcaccggct gtgatcagct acgcccagcaa tgatgtgaaa cctaaggcaa gcatcaagtt
601 ccaacaaact gttctggca caaaggcctgc accagccgtg gctttctata cttcttagagg

661 tccgtcaccg agctatccag gcatctaaa gccagatata atggcccctg ggtcactagt
721 ttttgctgct tggattccaa atactgctac agcccaaatt gtttgaata ccctcttgac
781 aagtgaatac aatatggttt ctggaacatc aatggcctgc cctcatgctg ctggtagc
841 tgctctcctt aaggcgcac accctgaatg gagtcagct gctatttagt ctgcaatgat
5 901 gactacagca aatcccttgg ataacacact aaatccaatc cggacaatg gtctaata
961 ttccacatct gttcacctt tagctatggg agccggccaa gttgatccta atcggcact
1021 tgcacccgtt ttgatttatg aaaccacccc acaagattat gtgagcctcc tctgactct
1081 gaacttcacc caaaacccaa tcctgtccat tacaagatca aaccgttaca gctgctccac
1141 ccctaattcct gatcttaact atccttcttt tattacttta cactacaaca caaatgcaac
10 1201 atttggtag acttttcaca ggactgtgac taacgttgg aagaagcgta caacttacaa
1261 ggccaagatc actgctcctc tagttctgt agtttgtc tcaccagaca cattggcctt
1321 cagaaagcag tatgaggcgc agagctacga gctcaattt gaggacaagc ctgtatggta
1381 agaaactgtt tcattttggg aacttgggg gattgaagaa aatggaaatc acactgtgag
1441 gagccctatt acagtgtcac cttccatgag taactttgtg tttatggta cacaataatt
15 1501 gataaaaatt tggatgtatc acaactgtgg gaataatcga cgtttatgaa cccagaataa
1561 gttgttttgtt cgtttcaac attatcataa aggacttggaa tcatgtgtgt tgattttctg
1621 caaaaaaaaaaaaaaaa aagtactctg cggtgataacc actgcttgcc ctatagtgag
1681 tcgtattag

20 The overlapping sequences SEQ. ID. NO. 6 and SEQ. ID. NO. 7 make it possible to deduce the complete sequence of the cDNA of sequence SEQ. ID. NO. 8 encoding for heterocarpine. The sequence SEQ. ID. NO. 8 is reproduced hereafter:

- SEQ. ID. NO. 8:

1 ctaatacgac tcactatagg gcaaggcgtg gtatcaacgc agagtacgac gggatgcccc
61 aagctaattc ttatctttt tctttctttt tggatgtgtt ttgtcaaaagc agcaatgagg
25 121 tcttaggaatg gtgttcttca tttattcctt ttcgttcttgc catggcttct gttggcggct
181 ctccatgcta actcaagttc ggatgagaga tcaacatata tagttcatat ggacaagacc
241 catatgccca aaaccccttc tagccccac cattggtaact cttcggtcgt tcgatccctc
301 aagtctacaa agccaaacccaa attaaatcgc cgtcgatcct caccacttct tgtataactct
361 tacgacaatg ctgctcatgg tttcagtgtca gttttatctc aacaggaact tgaaaactcta

421 aaaaagtctc caggttcgt ctca~~g~~tttat gccgataaga cagcgacact tgacaccacc
481 catacacctg aatttctctc cctgaatact gccaacgggt tgtggcctgc ttcaaagtat
541 ggtgaagata taattgttg~~g~~ t~~g~~tattgac agcgggtct ggccggagag t~~g~~aaagttat
601 aatgatgatg gtatggcgc tattcca~~a~~gc agatggaagg gagaatgtga agctggacaa
5 661 gagttcaatt cctccatgtg caactcaa~~a~~g cttattggag ct~~g~~atattt cgataagggt
721 atcattgcgg caa~~a~~c~~t~~c~~t~~gg gattaacatt agcatgaaat ctgccagaga tactatgggg
781 catgggactc acacatc~~c~~tc cacagttgct g~~g~~gaattatg tggatggcgt ttcattctt
841 ggctatgcta aaggtacagc aaaaggagtg gcaccacggg cgagagtggc tatgtacaag
901 gtcattttg acgaaggcg~~c~~ ctatgc~~a~~tct gatgttctt~~g~~ cc~~g~~gtatgga cgccgctatt
10 961 gctgatgg~~t~~ ttgatgtaat ttcaat~~a~~tca atggattt~~g~~ atgagacccc gttgtatgaa
1021 gatcctatag caattgc~~c~~tc attcg~~c~~tgct acagagaagg gcgtat~~g~~gt ctc~~a~~tcttca
1081 gcaggaaatg cagggcc~~a~~gc gctagg~~g~~agc ttgcacaatg gaatccc~~a~~tg gacgttaact
1141 gttcagctg gaaccattga ccgtt~~c~~attt gcaggcacta taactcttgg gagtggggaa
1201 accatcattg gatggacaat gttccc~~a~~g~~c~~ agtgctt~~a~~tg tagcagactt gca~~c~~actgctt
15 1261 tataacaaga ctta~~c~~tctgc atgcaactca actcgattat tatctcaact ccgaactgac
1321 gccatcatcg tatgc~~a~~aga agctgaagat tcggtatctg agcaaata~~t~~c t~~g~~tgtc~~a~~gt
1381 gcatcgaaca tt~~c~~ggggagc catattt~~g~~tt tcagattatg atgctgaatt att~~t~~gaactt
1441 ggtgg~~t~~gtga ctattc~~c~~ttgg t~~g~~t~~c~~gtgatt agc~~a~~ccaagg atgcac~~cc~~gc t~~g~~t~~g~~atc~~a~~gc
1501 tacgccagca atgatgt~~g~~aa acctaaggca agcatca~~a~~gt tccaacaaac t~~g~~ttctgggc
20 1561 acaaaggctg caccagg~~c~~gt ggctt~~c~~tat acttcttag~~g~~ gtccgtcacc gagctatcca
1621 ggc~~a~~tcttaa agccagat~~a~~t aatggccc~~c~~ g~~g~~tcact~~g~~ ttttgc~~t~~gc ttggattcca
1681 aatactgcta cagccccaaat tgg~~t~~ttgaat accctt~~c~~ga caagtgaata caatatgg~~t~~
1741 tctggaacat caatggc~~c~~tg ccctcatgct gctgg~~t~~gtag ctgctctc~~c~~t taagggc~~g~~ca
1801 caccctgaat ggagtgc~~a~~gc tgctattagg tctgcaatga tgactacagc aaatcc~~c~~ttg
25 1861 gataacacac taaatccaat ccgggacaat ggtcta~~a~~tca atttcacatc tgcttcac~~c~~ct
1921 ttagctatgg gagccggcca agttgat~~c~~c~~t~~ aatcgggcac ttgatc~~c~~ttgg tttgatttat
1981 gaaaccaccc cacaagatta t~~g~~t~~g~~agc~~c~~tc ctctgc~~a~~ctc tgaacttcac c~~cc~~aaaaccaa
2041 atcctgtcca ttacaagatc aaaccgttac agctgctcca ccccta~~a~~tcc tgatcttaac
2101 t~~a~~tcc~~t~~t~~t~~ttt ttattacttt acactacaac acaa~~a~~atg~~c~~aa catttgc~~t~~ca gactttc~~a~~c

2161 aggactgtga ctaacgttgg aggaagcgct acaacttaca aggccaagat cactgctcct
2221 ctaggttctg tagtttgtgt ctcaccagac acattggcct tcagaaagca gtatgagcag
2281 cagagctacg agctcactat tgagtacaag cctgatggtg aagaaactgt ttcatgggg
2341 gaacttggtt ggattgaaga aaatggaaat cacactgtga ggagccctat tacagtgtca
5 2401 cttccatga gtaactttgt gtttatgggt acacaataat tgataaaaat ttgttctgat
2461 cacaactgtg ggaataatcg acgtttatga acccagaata agttgttgg tcgtcttcaa
2521 cattatcata aaggacttga atcatgtgtg ttgattttct gaaaaaaaaaaaaaaa
2581 aaagtactct gcgttgatac cactgcttgc cctatagtga gtcgtattag

In the sequence SEQ. ID. NO. 8, an open reading frame is observed in the presence of
10 an initiation codon (ATG) encoding for an initiator methionine in position 115 and a stop codon (UAA) in position 2437. The polynucleotide containing the sequence encoding for heterocarpine corresponds to the sequence SEQ. ID. NO. 9, reproduced hereafter:

- SEQ. ID. NO. 9:

15 1 atgaggctca ggaatggtgt ttttcattta ttccctttcg ttcttgcatg gcttcgttgc
61 gcggctctcc atgctaactc aagttcggat gagagatcaa catatatagt tcataatggac
121 aagaccata tgcccaaaac cttctctagc ccccaccatt ggtactcttc ggtcgttcga
181 tccctcaagt ctacaaagcc aaccaaatta aatcgccgtc gatcctcacc acttcttgta
241 tactttacg acaatgtgc tcatggtttc agtgcagttt tatctcaaca ggaacttgaa
20 301 actctaaaaa agtctccagg tttcgctca gtttatgccg ataagacagc gacacttgac
361 accaccata cacctgaatt tctctccctg aataactgcca acggggttgta gcctgcttca
421 aagtatggtg aagatataat tggatgggtt attgacagcg gtgtctggcc ggagagtgaa
481 agttataatg atgatggat gggcgctatt ccaagcagat ggaagggaga atgtgaagct
541 ggacaagagt tcaattcctc catgtcaac tcaaagctta ttggagctag atatttcgat
25 601 aaggatca ttgcggaaa tcttggatt aacattagca tgaaatctgc cagagatact
661 atggggcatg ggactcacac atcctccaca gttgctggaa attatgtgga tggcgttca

721 ttctttggct atgctaaagg tacagcaaaa ggagtggcac cacgggcgag agtggctatg
781 tacaaggtca ttttgacga agggcgctat gcatctgatg ttcttgccgg tatggacgcg
841 gctattgctg atgggtttga tgtaatttca atatcaatgg gatttgatga gaccccgttg
901 tatgaagatc ctatagcaat tgccctcattc gctgctacag agaagggcgt agtggctca
5 961 tcttcagcag gaaatgcagg gccagcgcta gggagcttgc acaatggaat cccatggacg
1021 ttaactgttg cagctggAAC cattgaccgt tcatttgcAG gcactataAC tcttgggAGt
1081 ggggAAACCA tcattggATG gacaatgttC ccagccAGtG cttatgtAGC agacttgcCA
1141 ctgctttata acaagactta ctctgcatgc aactcaactc gattattatc tcaactccga
1201 actgacGCCA tcatcgatG cgaagaAGt gaagattcGG tatctgagca aatatctgtt
10 1261 gtcagtgcAT cgaacattcG gggagccATA tttgtttcAG attatgtatc tgaatttattt
1321 gaacttggtg gtgtgactat tcctggtgTC gtgattAGCA ccaaggatgc accggctgtg
1381 atcagctacg ccagcaatGA tgtgaaACCT aaggcaAGCA tcaagttCCA acaaACTgtt
1441 ctgggcacAA agcctgcACC agccgtggct ttctataCTT ctagaggTCC gtcaccgAGC
1501 tatccaggca tcttaaAGCC agatataatG gcccctgggt cactagTTT tgctgcttgg
15 1561 attccaaATA ctgctacAGC ccaaattgggt ttgaataACCC tcttgacaAG tgaataACAAT
1621 atggtttctg gaacatcaat ggctgcCCT catgctgctG gtgtagctGC tctccttaAG
1681 ggCGCACACC ctgaatggag tgcagctgCT attaggtctG caatgatgac tacagcaaAT
1741 cccttggata acacactAAA tccaatccGG gacaatggtC taatcaattt cacatctgCT
1801 tcacctttAG ctatggAGC cgGCCAAGt gatcctaATC gggcacttGA tcctggTTG
20 1861 atttatgaaa ccacCCcaca agattatgtG agcctcCTtC gcactctgAA ctccacCCAA
1921 aaccaaATCC tgcattAC aagatcaaAC cgttacAGt gctccacCCC taatcctgat
1981 cttaactatC cttctttat tactttacAC tacaacacAA atgcaacATT tggcAGACT
2041 tttcacAGGA ctgtgactAA cggtggAGGA agcgctacAA cttacaAGGC caagatcact

2101 gtcctctag gttctgttagt tagtgtctca ccagacacat tggccttcag aaagcagtag
2161 gagcagcaga gctacgagct cactatttag tacaaggctg atggtaaga aactgtttca
2221 ttggggAAC ttgtttggat tgaagaaaat ggaaatcaca ctgtgaggag ccctattaca
2281 gtgtcacctt ccatgagtaa ctttgtttt atgggtacac aataa

- 5 A protein of sequence SEQ. ID. NO. 10, composed of 774 amino acids and reproduced hereafter corresponds to the polynucleotide thus translated:

- SEQ. ID. NO. 10:

1 M R S R N G V L H L F L F V L A W L L L A A L H A N S S S D
31 E R S T Y I V H M D K T H M P K T F S S P H H W Y S S V V R
10 61 S L K S T K P T K L N R R R S S P L L V Y S Y D N A A H G F
91 S A V L S Q Q E L E T L K K S P G F V S V Y A D K T A T L D
121 T T H T P E F L S L N T A N G L W P A S K Y G E D I I V G V
151 I D S G V W P E S E S Y N D D G M G A I P S R W K G E C E A
181 G Q E F N S S M C N S K L I G A R Y F D K G I I A A N P G I
15 211 N I S M K S A R D T M G H G T H T S S T V A G N Y V D G V S
241 F F G Y A K G T A K G V A P R A R V A M Y K V I F D E G R Y
271 A S D V L A G M D A A I A D G V D V I S I S M G F D E T P L
301 Y E D P I A I A S F A A T E K G V V V S S S A G N A G P A L
331 G S L H N G I P W T L T V A A G T I D R S F A G T I T L G S
20 361 G E T I I G W T M F P A S A Y V A D L P L L Y N K T Y S A C
391 N S T R L L S Q L R T D A I I V C E E A E D S V S E Q I S V
421 V S A S N I R G A I F V S D Y D A E L F E L G G V T I P G V
451 V I S T K D A P A V I S Y A S N D V K P K A S I K F Q Q T V
481 L G T K P A P A V A F Y T S R G P S P S Y P G I L K P D I M
25 511 A P G S L V F A A W I P N T A T A Q I G L N T L L T S E Y N
541 M V S G T S M A C P H A A G V A A L L K G A H P E W S A A A
571 I R S A M M T T A N P L D N T L N P I R D N G L I N F T S A
601 S P L A M G A G Q V D P N R A L D P G L I Y E T T P Q D Y V

631 S L L C T L N F T Q N Q I L S I T R S N R Y S C S T P N P D
661 L N Y P S F I T L H Y N T N A T F V Q T F H R T V T N V G G
691 S A T T Y K A K I T A P L G S V V S V S P D T L A F R K Q Y
721 E Q Q S Y E L T I E Y K P D G E E T V S F G E L V W I E E N
5 751 G N H T V R S P I T V S P S M S N F V F M G T Q

Example 2: preparation of the complete cDNA encoding for heterocarpine for producing recombinant heterocarpine:

2.1) Preparation of the RNAs from *Pilocarpus Heterophyllus* cell culture:

- The cells in culture are preserved at -80°C before the stages of total RNA extraction.
10 The total RNA extraction is based on a technique described in the scientific literature (Chomczynski and Sacchi, *Anal. Biochem.* (1987), **162**, 156) using Trizol reagent (Gibco/BRL). The quality of the RNAs thus extracted is analyzed on 1% agarose gel in the presence of ethidium bromide.

2.2) Reverse transcription from the RNAs:

- 15 The total RNAs are reverse transcribed with Oligo(dT) primers using Superscript® reverse transcriptase as suggested in the manufacturer's (Gibco/BRL) manual.

2.3) Polymerase chain reaction (PCR) on the products obtained from reverse transcription :

- 20 The amplification of the cDNA of heterocarpine was carried out by polymerase chain reaction (PCR) on the products of reverse transcription using the primers Fwd2 and Rev2 of respective sequences SEQ. ID. NO. 11 and SEQ. ID. NO. 12.

The sequences SEQ. ID. NO. 11 and SEQ. ID. NO. 12 are the following:

- SEQ. ID. NO. 11:

5'-GGA TCC GAG GTC TAG GAA TGG TGT TCT TCA-3'

25 - SEQ. ID. NO. 12:

5'-GGG CTC GAG TTG TGT ACC CAT AAA CAC AAA GTT ACT CAT GG-3'

- 30 The primer Fwd2 corresponds to the nucleotides 118 to 140 of SEQ. ID. NO. 8 and includes a BamH1 site (underlined) and three additional nucleotides in the 5' part in order to facilitate the cloning. The primer Rev2 corresponds to the sequence complementary to the region containing the nucleotides 2405 to 2436 of SEQ. ID. NO.

8 and includes an Xhol site (underlined) and three additional nucleotides in the 5' part in order to facilitate the cloning.

The reaction conditions include 50 ng of the cDNA products of the reverse transcription reaction described above, 0.2 µM of Fwd2 (SEQ. ID. NO. 11) and of Rev2 (SEQ. ID. NO. 12), 200 µM dNTPs, 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM Mg(Oac)₂, 3.75 µg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, and 0.5 U Taq DNA polymerase in a final volume of 50 µl. The PCR reactions are carried out in a Perkin-Elmer 9700 thermocycler using the following thermal cycle parameters: 5 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 72°C, 5 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 70°C for 10 seconds, and a polymerase extension at 72°C for 3 minutes and finally 25 cycles comprising a denaturation at 94°C for 5 seconds, a hybridization of the primers at 68°C for 10 seconds, and a polymerase extension at 72°C for 3 minutes.

The products obtained by PCR are separated on 1% agarose gel and visualized using ethidium bromide staining. An approximately 2.3 kb band is obtained.

The nucleic acid sequence of the PCR product is verified using an automatic sequencer and corresponds to the sequence SEQ. ID. NO. 9 having artificially undergone deletion of the initiation codon ATG in order to allow the expression of recombinant heterocarpine from the pQE-TriSystem (Qiagen) phase vector with the BamH1 site as well as deletion of the stop codon in order to preserve the phase translation to protein and thus allow the synthesis of an 8xHis sequence in the C-terminal region of heterocarpine. This sequence corresponds to the sequence SEQ. ID. NO. 13 reproduced hereafter:

- SEQ. ID. NO. 13:

25 1 gggggatccg aggtcttagga atgggtttct tcatttattc ctttcgttcc ttgcattggct
61 tctgttggcg gctctccatg ctaactcaag ttcggatgag agatcaacat atatagttca
121 tatggacaag acccatatgc cccaaacctt ctctagcccc caccatttgt actcttcggt
181 cgttcgatcc ctcaagtcta caaagccaac caaattaaat cgccgtcgat cctcaccact
241 tcttgatac tcttacgaca atgctgctca tggtttcagt gcagtttat ctcaacagga
30 301 acttgaaact ctaaaaaagt ctccagggtt cgtctcagtt tatgccgata agacagcgac
361 acttgacacc acccatacac ctgaatttct ctccctgaat actgccaacg gggtgtggcc
421 tgcttcaaag tatggtaag atataattgt tggtgttatt gacagcggtg tctggccgga

481 gagtgaaagt tataatgatg atggtatggg cgctattcca agcagatgga agggagaatg
541 tgaagctgga caagagttca attcctccat gtgcaactca aagcttattg gagctagata
601 tttcgataag ggtatcattg cggcaaatcc tgggattaac attagcatga aatctgccag
661 agatactatg gggcatggga ctcacacatc ctccacagtt gctgggaatt atgtggatgg
5 721 cgtttcattc tttggctatg ctaaaggtaac agcaaaaagga gtggcaccac gggcgagagt
781 ggctatgtac aaggtcattt ttgacgaagg gcgcstatgca tctgatgttc ttgcccggtat
841 ggacgcggct attgctgatg gtgttgatgt aatttcaata tcaatggat ttgatgagac
901 cccgttgtat gaagatccta tagcaattgc ctcattcgct gctacagaga agggcgttagt
961 ggtctcatct tcagcagggaa atgcagggcc agcgctaggg agcttgcaca atggaatccc
10 1021 atggacgtta actgttgcag ctggaaccat tgaccgttca tttgcaggca ctataactct
1081 tgggagtgaaa gaaaccatca ttggatggac aatgttccca gccagtgctt atgttagcaga
1141 cttgccactg ctttataaca agacttactc tgcatgcaac tcaactcgat tattatctca
1201 actccgaact gacgccatca tcgtatgcga agaagctgaa gattcggat ctgagcaaatt
1261 atctgttgc agtgcattcga acattcgggg agccatattt gttcagatt atgatgctga
15 1321 attatggaa cttgggtggtg tgactattcc tgggtcgatg attagcacca aggtgcacc
1381 ggctgtgatc agctacgcca gcaatgatgt gaaacctaag gcaagcatca agtccaaaca
1441 aactgttctg ggcacaaaagc ctgcaccagc cgtggcttc tatacttcta gaggtccgtc
1501 accgagctat ccaggcatct taaagccaga tataatggcc cctgggtcac tagttttgc
1561 tgcttggatt ccaaatactg ctacagcccc aattgggtttg aataccctct tgacaagtga
20 1621 atacaatatg gtttctggaa catcaatggc ctgcctcat gctgctggatg tagctgtct
1681 ccttaagggc gcacaccctg aatggagtgc agctgctatt aggtctgcaa tgatgactac
1741 agcaaatccc ttggataaca cactaaatcc aatccgggac aatggtctaa tcaatttcac
1801 atctgcttca ctttagcta tgggagccgg ccaagttgat cctaattcggg cacttgcatt
1861 tggtttgatt tatgaaacca ccccacaaga ttatgtgagc ctcctctgca ctctgaactt
25 1921 cacccaaaac caaatcctgt ccattacaag atcaaaccgt tacagctgct ccacccctaa
1981 tcctgatctt aactatcctt ctttattac tttacactac aacacaaaatg caacatttgc
2041 tcagactttt cacaggactg tgactaacgt tggaggaagc gctacaacctt acaaggccaa
2101 gatcactgct cctctaggat ctgttagttatg tgtctcacca gacacattgg ctttcagaaaa
2161 gcagtatgag cagcagagct acgagctcac tattgagttac aaggcctgatg gtgaagaaac

2221 tgtttcattt ggggaacttg tttggattga agaaaatggg aatcacactg tgaggagccc
2281 tattacagtg tcaccttcca tgagtaactt tgtgttatg ggtacacaac tcgagccc

This sequence encodes for a protein of sequence SEQ. ID. NO. 14 reproduced hereafter:

5 1 M A I S R E L V D P R S R N G V L H L F L F V L A W L L A
31 31 A L H A N S S S D E R S T Y I V H M D K T H M P K T F S S P
61 61 H H W Y S S V V R S L K S T K P T K L N R R R S S P L L V Y
91 91 S Y D N A A H G F S A V L S Q Q E L E T L K K S P G F V S V
121 121 Y A D K T A T L D T T H T P E F L S L N T A N G L W P A S K
10 10 151 Y G E D I I V G V I D S G V W P E S E S Y N D D G M G A I P
181 181 S R W K G E C E A G Q E F N S S M C N S K L I G A R Y F D K
211 211 G I I A A N P G I N I S M K S A R D T M G H G T H T S S T V
241 241 A G N Y V D G V S F F G Y A K G T A K G V A P R A R V A M Y
271 271 K V I F D E G R Y A S D V L A G M D A A I A D G V D V I S I
15 15 301 S M G F D E T P L Y E D P I A I A S F A A T E K G V V V S S
331 331 S A G N A G P A L G S L H N G I P W T L T V A A G T I D R S
361 361 F A G T I T L G S G E T I I G W T M F P A S A Y V A D L P L
391 391 L Y N K T Y S A C N S T R L L S Q L R T D A I I V C E E A E
421 421 D S V S E Q I S V V S A S N I R G A I F V S D Y D A E L F E
20 20 451 L G G V T I P G V V I S T K D A P A V I S Y A S N D V K P K
481 481 A S I K F Q Q T V L G T K P A P A V A F Y T S R G P S P S Y
511 511 P G I L K P D I M A P G S L V F A A W I P N T A T A Q I G L
541 541 N T L L T S E Y N M V S G T S M A C P H A A G V A A L L K G
571 571 A H P E W S A A A I R S A M M T T A N P L D N T L N P I R D
25 25 601 N G L I N F T S A S P L A M G A G Q V D P N R A L D P G L I
631 631 Y E T T P Q D Y V S L L C T L N F T Q N Q I L S I T R S N R
661 661 Y S C S T P N P D L N Y P S F I T L H Y N T N A T F V Q T F
691 691 H R T V T N V G G S A T T Y K A K I T A P L G S V V S V S P
721 721 D T L A F R K Q Y E Q Q S Y E L T I E Y K P D G E E T V S F
30 30 751 G E L V W I E E N G N H T V R S P I T V S P S M S N F V F M
781 781 G T Q L E H H H H H H H H H

Example 3: production of recombinant heterocarpine by bacteria:

The part of the cDNA encoding for heterocarpine is inserted at the BamH1/Xhol sites of the pQE-TriSystem (Qiagen) expression vector and is expressed using *E. coli* M15 bacteria as host bacteria. 20 ml of LB medium containing 100 µg/ml of ampicillin and

25 µg/ml of kanamycin are inoculated and the bacteria brought to 37°C under stirring for 12 hours. From this culture, 1 litre of LB medium containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin is inoculated and the bacteria are stirred at 37°C until an optical density of 0.6 at 600 nm is obtained.

- 5 The expression of the recombinant heterocarpine is produced by the addition of IPTG at a final concentration of 1 mM over 4 to 5 hours. The bacteria are then recovered by centrifugation at 4000 x g for 20 minutes then frozen in liquid nitrogen. The pellet is then thawed in ice for 15 minutes and suspended in a pH 8.0 lysis buffer composed of 50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole in the presence of 1 mg/ml of lysozyme for 30 minutes in ice. The lysis is finally completed by a sonication stage and the debris eliminated by centrifugation. The lysate (4 ml) thus clarified is mixed with 1 ml of a nickel matrix suspension and stirred at 4°C for 60 minutes. All of the reaction medium is placed in a column, washed in the presence of 50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole. The heterocarpine is finally eluted with 4 x 0.5 ml of buffer constituted by 50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole.
- 10
- 15

Example 4: production of recombinant heterocarpine by cells from insects infected by the baculovirus:

The part of the cDNA encoding for heterocarpine is inserted at the BamH1/Xho1 sites of the pQE-TriSystem (Qiagen) expression vector. The pQE-TriSystem vector contains the viral sequences of *Autographa californica nuclear polyhedrosis virus* (AcNPV) allowing homologous recombination. The recombinant baculovirus containing the heterocarpine sequence is prepared by co-transfection of the pQE-TriSystem vector with the genomic linearized DNA of the baculovirus in sf9 or sf21 insect cells established from ovarian tissues of the *Spodoptera frugiperda* larva. The transfected cells are washed with phosphate buffer and collected by centrifugation at 1000 x g for 5 minutes. The pellet is suspended in a pH 8.0 lysis buffer composed of 50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole. The lysis is finally completed by a sonication stage and the debris eliminated by centrifugation. The lysate (4 ml) thus clarified is mixed with 200 µl of a nickel matrix suspension and stirred at 4°C for 60 minutes. All of the reaction medium is placed in a column, washed in the presence of 50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole. The heterocarpine is finally eluted with 4 x 0.5 ml of buffer constituted by 50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole.

Example 5: production of recombinant heterocarpine by mammal cells:

The part of the cDNA encoding for heterocarpine is inserted at the BamH1/Xhol sites of the pQE-TriSystem (Qiagen) expression vector. The pQE-TriSystem vector contains the activating sequences of the cytomegalovirus (CMV) fused to chicken beta-actin promoter allowing a very significant heterologous expression. Human embryo kidney (HEK-293) cells are cultured in DMEM medium (Dulbecco's Modified Eagle's Medium) containing 100 U/ml of penicillin and 100 µg/ml of streptomycin sulphate, complemented with 10% foetal calf serum. The cells are sub-cultured 24 hours before the transfection protocol allowing normal metabolism of the cells and better transfection efficiency. The transfection of 1 µg of pQE-TriSystem containing the cDNA encoding for heterocarpine was carried out using Effectene® reagent according to the manufacturer's (Qiagen) recommendations.

The transfected cells are washed with phosphate buffer and collected by centrifugation at 1000 x g for 5 minutes. The pellet is suspended in a pH 8.0 lysis buffer composed of 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole in the presence of 0.05% Tween® 20. The lysis is finally completed by a sonication stage and the debris eliminated by centrifugation. The lysate (4 ml) thus clarified is mixed with 200 µl of a nickel matrix suspension and stirred at 4°C for 60 minutes. All of the reaction medium is placed in a column, washed in the presence of 50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole. The heterocarpine is finally eluted with 4 x 0.5 ml of buffer constituted by 50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole.

Example 6: measurement of the binding to the human GHRH receptor:

*Stable transfections of the human GHRH receptor (*hGHRH-R*):*

HEK-293 human embryo kidney cells (a cell line developed by Dr. Stuart Sealfon, Mount Sinai Medical School, New York, New York) expressing the human GHRH receptor in stable manner were obtained from Dr. Kelly Mayo (Northwestern University, Chicago, IL).

Cell culture and membrane preparation:

The HEK-293 cells transfected in a stable manner with the human GHRH receptor described above are cultured in DMEM (Dulbecco's Modified Eagle's Medium, high glucose content; supplied by Life technologies) supplemented with 0.4 mg/ml of G418 (Life technologies) in the presence of 10% foetal calf serum and 4 mM of L-glutamine (Life Technologies). The cells are homogenized in buffer A containing 50 mM HEPES (pH 7.4), 5 mM of magnesium chloride (MgCl₂), 2 mM of ethyleneglycol-bis(2-amino-ethyl)-N,N,N',N'-tetraacetic acid (EGTA) and 50 µg/ml of bacitracin then

subjected to sonication in the same buffer A. The cells thus homogenized are centrifuged at 4°C at 39,000 g for 10 minutes, suspended in the buffer A and re-centrifuged at 4°C at 40,000 g for 10 minutes. The total membrane proteins are quantified by Bradford's technique. The pelleted membranes are thus stored at -80°C
5 for later use.

Competitive binding test on hGHRH-R:

The membranes of the HEK-293 cells transfected in a stable manner with the human GHRH receptor are diluted to a concentration of 100 µg/ml in reaction buffer
10 containing 50 mM HEPES (pH 7.4), 5 mM of MgCl₂, 2 mM of EGTA, 50 µg/ml of bacitracin and 0.5% bovine serum albumin (BSA). The membranes are incubated with 0.05 nM of [¹²⁵I]GHRH(1-44 amide) (Amersham) in a final volume of 200 µl in the presence of increasing concentrations of heterocarpine for 2 hours at 23°C. The reaction
15 is stopped by rapid filtration on GF/C 96-well filters 0.1% pre-loaded with polyethylenimine. The filters are then washed three times at 4°C with washing buffer containing 50 mM Tris (pH 7.4) using a Packard 96-well filtration station. The filters thus dried are submerged in 20 µl of scintillation cocktail (Microscint O, Packard) and are subjected to counting by Topcount (Packard). The non-specific activity is determined in the presence of 100 nM of hGHRH. A dose-response curve is generated
20 for hGHRH (0.001 nM-100 nM) and makes it possible to determine the inhibitory concentration IC₅₀ of protein / polypeptide at which 50% human GHRH does not bind to human GHRH receptor.